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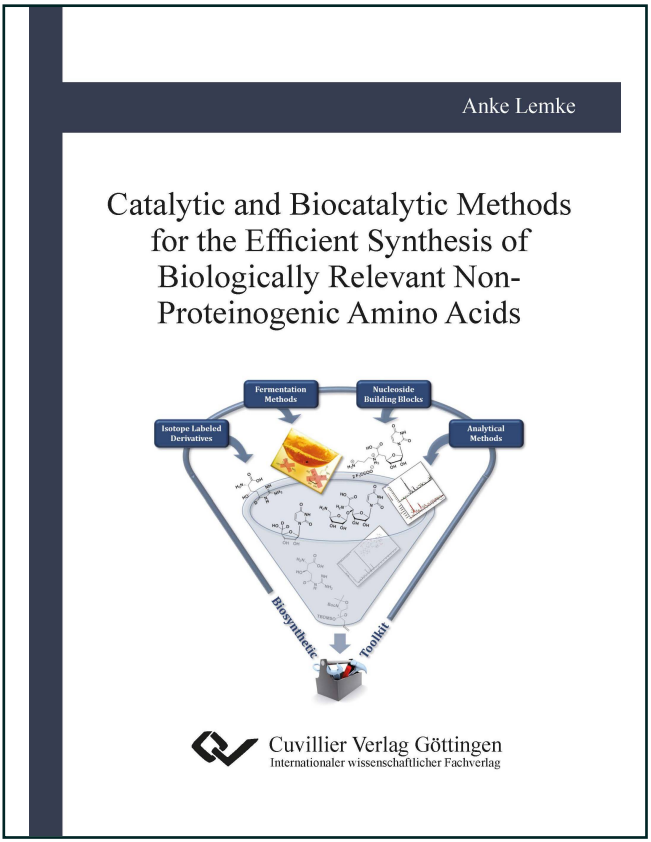
Catalytic and Biocatalytic Methods for the Efficient Synthesis of Biologically Relevant Non-Proteinogenic Amino Acids



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1 Introduction

1.1 Antibiotics

Penicillin G®, *Vancomycin*®, and *Amoxicillin*® are drug names that probably everybody has read on a prescription once, or at least heard of. These trade names of antibiotics illustrate only 3 of the 80 different therapeutically established antibiotics in Germany.[2] Thus, it is not surprising that antibiotics still belong to the most prescribed drugs. The estimated total consumption of antibiotics in human medicine in Germany lies between 250-300 t per year. A quantum of 85% were prescribed in the outpatient care (GERMAP 2008).[3] In 2002, the global market was estimated at US $25 billion, and 6 antibiotics were topping US $1 billion each.[4] The mainstay of antibiotic scaffolds, including the 6 bestsellers, was represented by 3 structural classes for decades: the β-lactams (e.g. penicillin, amoxicillin, ceftriaxone), the macrolides (e.g. azithromycin, clarithromycin) and the quinolones (e.g. ciprofloxacin, levofloxacin). Additional structural classes are described by sulfonamides, polyketides (e.g. tetracyclin), glycopeptides (e.g. vancomycin), streptogramines, oxazolidines and lipopetides (e.g. daptomycin).[5-6]

The incredibly fast rise of antibiotics started in the early 1940s when the demand for a cure against wound infections increased during the Second World War. The first clinically used antimicrobial drug was the prominent penicillin, which was isolated from the mold *Penicillium notatum* by *Florey* and *Chain* in 1940.[7] It had already been discovered by *Fleming* in 1928 though.[8] Although the new hyped wonder drug was the beginning of the golden age of microbiology and led to the discovery of numerous new antimicrobially active substances,[9] the known curative effect of molds goes way back to Chinese medicine in 1000 B.C., when mold-cultured soybean-curd was used to cure skin infections.[10] Moreover, the Middle American Indians used to treat purulent inflammations with wild mushrooms,[10] and in the Hashemite Kingdom of Jordan red soil is still used today as an inexpensive alternative to antibiotics.[11] But despite these and other numerous anecdotes about the occurrences of antibiotic-like effects from all over the world,[12] the first scientific report of antimicrobial activity did not appear until 1877 when *Pasteur* observed an antagonism between bacteria in the same culture medium.[13]

But when do we call a compound antibiotic, and how do antibiotics work? The word 'antibiosis' was first used by *Vuillemin* in 1889 to describe the concept of one active organism destroying the life of the passive one to maintain its own life.[14] The word 'antibiotic' was primarily defined by *Waksman* with 'antibiosis' meaning the inhibition of growth of one organism by another.[15] While in *Waksman's* definition of 'antibiotic', only secondary metabolites of bacteria and certain mushroom species were included, nowadays,

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the term also covers entirely synthetic compounds without a natural lead structure, which are used in the treatment of bacterial infectious diseases.

Antibacterially active substances can mainly act in two different ways: bacteriostatic (limiting the growth of bacteria) or bactericidal (killing the bacteria). In this process, they can target different essential functions in bacteria, like the bacterial cell wall synthesis, DNA- and RNA-replication, bacterial protein synthesis and folic acid metabolism (see chapter 2.1).[4] The most successful antibiotics of our time hit these four classical targets only, and they only offer a few different modes of action. In contrast, there are approximately 200 conserved essential proteins in bacteria. The number of the currently exploited targets is very small though, and it still bears potential for the discovery of new antibiotic lead structures and modes of action.[16]

1.2 Antibiotic Resistance and the Critical Need for New Antibiotics

Due to the expansive discovery of new antibiotics in the 20th century, life threatening diseases or epidemics like cholera, diphtheria, pneumonia, or tuberculosis seemed more or less under control. But if the major infectious diseases of the 20th century are defeated, where is the need for the laborious and expensive discovery of new antibiotics? While the pharmaceutical industry found their answer in a decreasing antibiotic research, which resulted in an innovation gap between 1960 and 2000 (Figure 1.1),[4,12] the threat of antibiotic resistance aroused. The first report of antibacterial resistance, represented by the penicillinase, appeared simultaneously with the introduction of penicillin into the clinical market.[17]

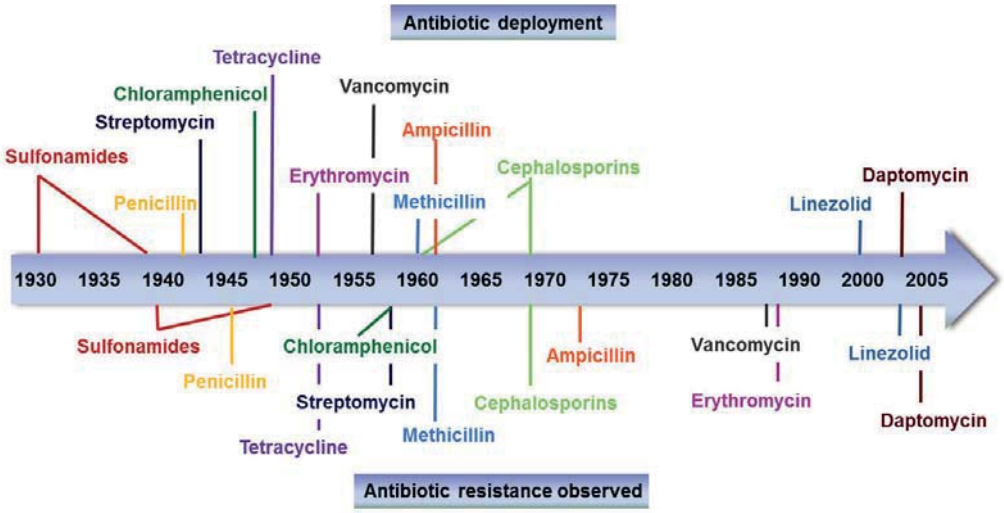

Figure 1.1: Timeline of the antibiotic deployment and the emerged resistance (adapted from: A. E. Clatworthy et al., *Nat. Chem. Biol*. **2007**, *3*, 541).[18]

Penicillinase is an enzyme of the β-lactamase family and hydrolyzes benzylpenicillin. This enzymatic transformation of penicillin describes only one of the six major mechanisms of antibiotic resistance (see also Figure 2.1). The other five mechanisms are a modification of

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the molecular target,[19] an active efflux from the cell interior,[20] a reduced entry of the compound due to alterations of penetration barriers,[21] finding a bypass for the inhibited sequence, or an increase of the production of the target metabolite.[16] The reason for the development of such mechanisms is evolutionary pressure, which leads to the selection of the resistant mutated organisms. Not only was the introduction of penicillin directly followed by its observed resistance. Nearly every antibiotic that had been clinically introduced entailed a significant resistance only a few years later (Figure 1.1).[18] Approximately 70% of the hospital-acquired infections are resistant to one or more antibiotics.[2,18] In this context, the occurrence of more and more multidrug-resistant bacteria is another alarming fact. The methicillin-resistant *Staphylococcus aureus* (MRSA)[22] and the vancomycin-resistant *Enterococcus faecium* (VRE) are only the most prominent examples. Multidrug resistance results from the ability of bacteria to transfer genetic resistance traits not only among their own, but also among different species. This horizontal gene transfer is mainly accomplished through transduction (via bacteriophages), conjugation (via plasmids and conjugative transposons), and transformation (via incorporation into the chromosome of DNA or plasmids).[12,23]

The very rapidly spreading multidrug resistance presents a new challenge to modern antibiotic research, in particular because in the last decades only a few new antibiotics were introduced into the clinical market.[24] These therapeutic agents are mainly based on the established scaffolds and are therefore missing new modes of action and the potential for clinical use. This is particularly critical if we think of extensively drug resistant (XDR) bacteria like *Mycobacterium tuberculosis*, which can be resistant to most antibiotics with classical targets.[25] These so called 'superbugs' are sparking fear for public health issues, especially since the latest news headlines announce: "*Superbugs: A ticking time bomb*" (CBS News),[26] or "*Europe 'losing' superbugs battle*" (BBC News Health).[27] Titles from fanatic blogs, such as "*How medicine is killing us all: Antibiotics, superbugs and the next global pandemics*" (NaturalNews.com),[28] even illustrate an apocalyptic scenario. But are we really on a critical edge of a post-antibiotic era, and the pharmaceutical industry is leading us there as some newspapers and maniacs want to make us believe?

Of course, due to a saturated antibiotic market in the 1960s and a high financial risk within antibacterial drug discovery, most of the large pharmaceutical companies and many biotechnology companies have left the area, leaving a gap in innovative strategies for today. But with the achievement of the first completely sequenced bacterial genome in 1995, several companies moved back into the antibacterials' area, hoping to unveil a whole treasure trove of new targets by a genomic-derived, target-based screening approach. Despite the promising identification of a whole new bunch of essential genes, the desired breakthrough could not be achieved, missing optimized lead structures suitable for clinical trials.[29] This example shows that there is not only an urgent need for new antibiotics, but also for new or improved approaches in antibacterial discovery research.

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The limited scientific resources are not the only big problem in the fight against antibiotic resistance though. Antibiotic misuse, poor hygienic standards in hospitals, and non-controllable release into the environment by households and animal husbandry are further problems to solve. Starting points here are standardized regulations in antibiotic usage and hygiene and awareness of educational responsibility.[30-31] Reports like the GERMAP 2008 about the antibiotic usage in Germany[3] and the 'Deutsche Antibiotika-Resistenzstrategie' (DART) are first steps into the right direction.[32] From 2008 to 2014, the DART initiative has invested €80 million in different projects for antibiotic research. Such investigations are hopefully enhancing the attractiveness for companies to reenter the antibiotic market or to increase their efforts in the antimicrobial research field. In the context of the combat against biological terrorism, the US Health and Human Services Department announced an agreement with *GlaxoSmithKline* this summer (2013), with the potential of as much as $94 million funding under the so called '10 x '20 Initiative'. The aim is to create a "sustainable global drug research and development enterprise with the power in the short term to develop 10 new, safe, and efficacious systemically administered antibiotics by 2020".[33] Having the effect of the antibiotic research progress of the Second World War in mind, such governmental support might enormously push the antibiotic innovation field.
Looking at the FDA approvals of antimicrobial drugs since 1998 (Table 1.1),[33] the scenario of a post-antibiotic era with antibiotic research as a lost cause seems unlikely. However, only a few of the approved drugs display new mechanisms, such as linezolide (oxazolidines) with binding to the ribosomal 50S subunit, daptomycin (lipopeptides) by membrane depolarization, followed by a disturbed bacterial ionic management, tigecyclin (glyciclines) with the ability to subvert common tetracycline resistance, and telavancin (glycopeptides) exerting an additional mode of action by depolarization and permeabilization of the bacterial membrane.[34]

Antibacterial	Year of FDA approval	Novel mechanism?
Rifapentine	1998	No
Quinupristin/Dalfopristin	1999	No
Moxifloxacin	1999	No
Gatifloxacin	1999	No
Linezolid	2000	Yes
Cefditoren pivoxil	2001	No
Ertapenem	2001	No
Gemifloxacin	2003	No
Daptomycin	2003	Yes
Telithromycin	2004	No
Tigecyclin	2005	Yes
Doripenem	2007	No
Telavancin	2009	Yes
Ceftaroline fosamil	2010	No

Table 1.1: Systemic antibacterial drug approvals since 1998.[33]

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Since 2007, only two systemic drugs have been approved. There are still potential candidates waiting for approval, but the pipeline is nearly dried nowadays. But where do new antibiotic lead structures come from, and what are promising strategies in antimicrobial research? Developing new antibiotics on established scaffolds with highly specifically improved properties is basically a good strategy. Nevertheless, these derivatives are more likely to enter a resistance stage that is critical for clinical use and are therefore still reflecting the urgent need for innovative strategies.

There are multiple strategies away from traditional antibiotic pathways, which are currently discussed, such as the design of antibacterial peptides,[35] modulating immunity, targeting virulence factors, the use of bacteriophages, prodrug concepts, and new delivering methods, to name only a few.[12,34] New creative strategies always bear the danger of failing though, as the 'genomic disaster' has proven.[29] Especially with new targets at hand, a large screening library with chemical diversity is essential. In this context, natural product leads still bear the highest potential of furnishing an active antimicrobial because they offer cellular permeability and defined structures for specific interactions with proteins. These in-nature-established pharmacodynamic properties are hard to design de novo. 'Back to the roots' is another promising guiding principle right now.[36] Searching for new natural lead structures in underexploited new areas, such as marine sediments or old areas like soil from all over the world, can deliver a whole bunch of structurally diverse compounds. In addition, quite old microbiological methods, like whole-cell assays, are rediscovered in modern research. Even the screening and improvement of fermentation conditions could deliver secondary metabolites that are only produced under a certain pH value or by the addition of special nutrients.[37] In this context, the activation of biosynthetic gene clusters, which are silent under standard laboratory conditions, is another interesting strategy.[38] This approach, also known as genome mining, bears the potential to discover numerous novel secondary metabolites.

Overall, there are still several promising antibacterial drugs with novel mechanisms of action in development.[37] But as new types of targets are emerging, it is more important than ever to take advantage of the large portfolio of biotechnological techniques, better knowledge of bacterial genetic function and the chemistry of natural lead structures, and to create an effective interdisciplinary field of research. This should lay the foundations for the development of effective antibiotics addressing new or clinically non-established targets. Promising key features of these antibacterially active compounds are often non-proteinogenic amino acids offering an extended chemical diversity and promising prospects in drug development.

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2 Literature Review

2.1 Clinically Established Antibiotic Classes and Their Targets

In order to understand the mode of action of certain antimicrobial substances and antibiotic classes, the classical antibiotic targets will be explained: interference with bacterial cell wall synthesis (a), DNA- and RNA-replication (b), bacterial protein synthesis (c) and folic acid metabolism (d) (Figure 2.1).[4]

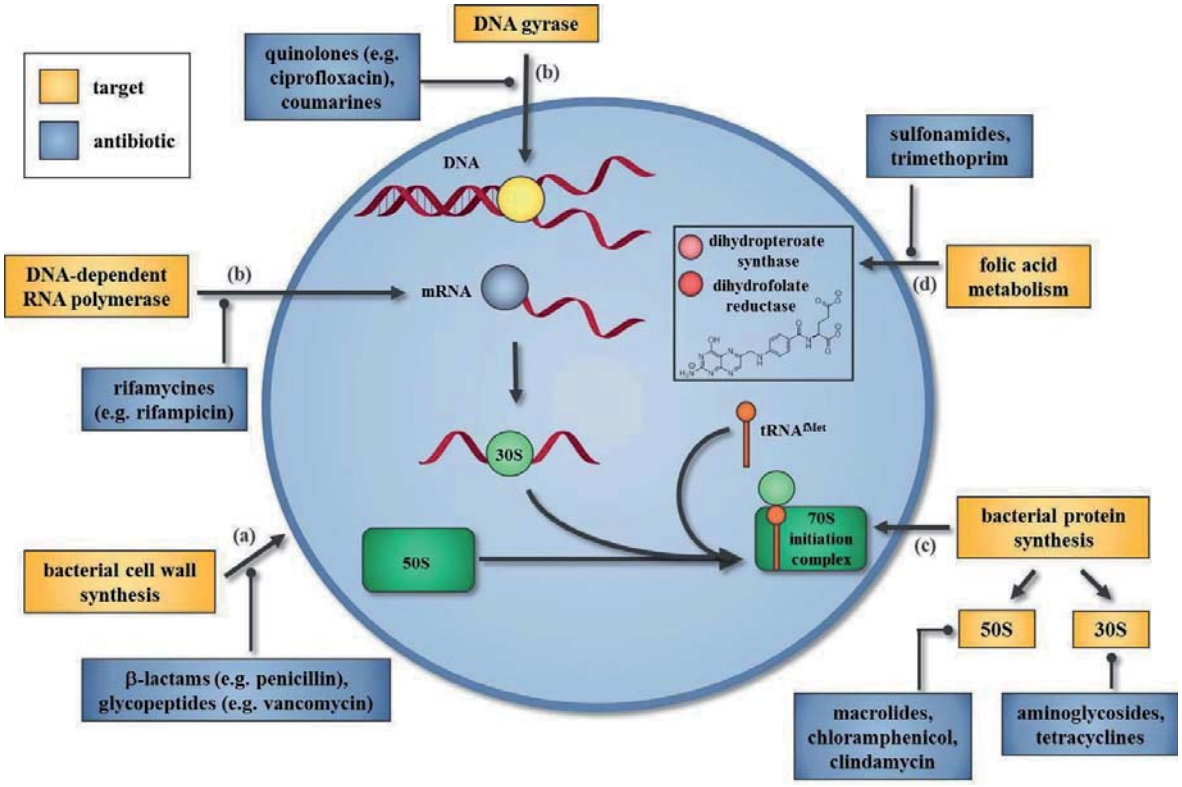

Figure 2.1: The four classical targets of established antibiotics (adapted from: K. Lewis et al., *Nat. Rev. Drug Discov.* **2013**, *12*, 371).[16]

In contrast to mammalian cells, folic acid biosynthesis is essential for bacterial survival. By acting as alternative substrates, structural analogues like sulfonamides inhibit the key enzyme dihydropteroate synthase. Another antibiotic targeting folate metabolism is trimethoprim, a 2,4-diaminopyrimidine, with the ability of selective inhibition of dihydrofolate reductase, which catalyzes the reduction of dihydrofolate to the crucial cofactor tetrahydrofolate.[39] As distinct binding sides of the ribosomal RNA subunits 50S and 30S provide the potential to block multiple steps in protein biosynthesis, numerous antimicrobial compounds, such as aminoglycosides, macrolides, tetracyclines, chloramphenicol, and clindamycin, were developed using this mode of action.[39-42] Quinolones, a well-established class of antibiotics (e.g. ciprofloxacin), inhibit DNA gyrase,

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which controls the topology of DNA. While promising DNA supercoiling inhibitors with new modes of action (e.g. coumarines) are still in the pipeline,[43] the DNA-dependent RNA polymerase, a major enzyme in the regulation of prokaryotic gene expression, remains quite underexploited in contrast, as it is only targeted by one class of clinically used antibiotics, the rifamycines (e.g. rifampicin).[39] The most established target for antibiotics in clinical use still remains the formation of the bacterial cell wall. In this context, the β-lactam antibiotics (e.g. penicillin) and glycopetides (e.g. vancomycin), two of the 'early-stage' antibiotic classes, are two of the first known inhibitors. Peptidoglycan, the essential cell wall building block, is a three-dimensional meshwork of peptide-cross-linked sugar polymers.[44] The interference with its biosynthesis or structure results in the loss of cell shape and integrity, followed by an inevitable bacterial death.[45] Peptidoglycan biosynthesis can be divided into three distinctive stages (**i**)-(**iii**) (Figure 2.2).

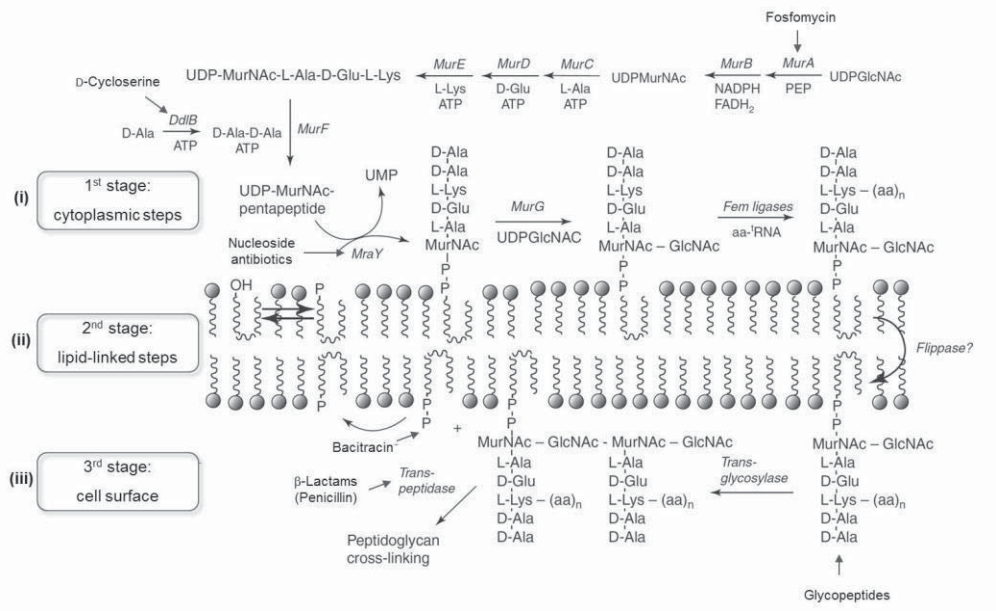

Figure 2.2: The peptidoglycan biosynthetic pathway showing sites of action of natural product inhibitors (adapted from: T. D. H. Bugg et al., *Trends Biotechnol.* **2011**, *29*, 168).[46]

The cytoplasmic steps which represent the first stage of peptidoglycan biosynthesis (**i**), lead from UDP-*N*-acetyl-glucosamine (Glc*N*Ac) to the peptidoglycan monomer UDP-*N*-acetyl-muramic acid (Mur*N*Ac) pentapeptide.[47-49] The second stage (**ii**) can be described as lipid-linked steps which involve the lipid carrier undecaprenyl phosphate.[50] After linking the monomer to the membrane, it is transferred to the cell surface, where, in a third stage (**iii**), it is polymerized and cross-linked to the pre-existing cell wall.[47,51] While well-established antibiotics like glycopeptides and β-lactams inhibit the late extracellular steps of the third stage, there are only few known antibacterial drugs in clinical use (D-cycloserine, fosfomycin, bacitracin) that target biosynthetic steps in stage one and two.[39] Due to this fact, bacterial cell wall assembly still remains an attractive target for the development of new antimicrobial compounds.[46]

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2.2 MraY as Target for Nucleoside Antibiotics

One target in the peptidoglycan biosynthetic pathway for which no clinically established inhibitor exists is the formation of lipid I from UDP-Mur*N*Ac pentapeptide (Figure 2.3). This reaction is catalyzed by the enzyme translocase I (MraY), which is integrated into the membrane.

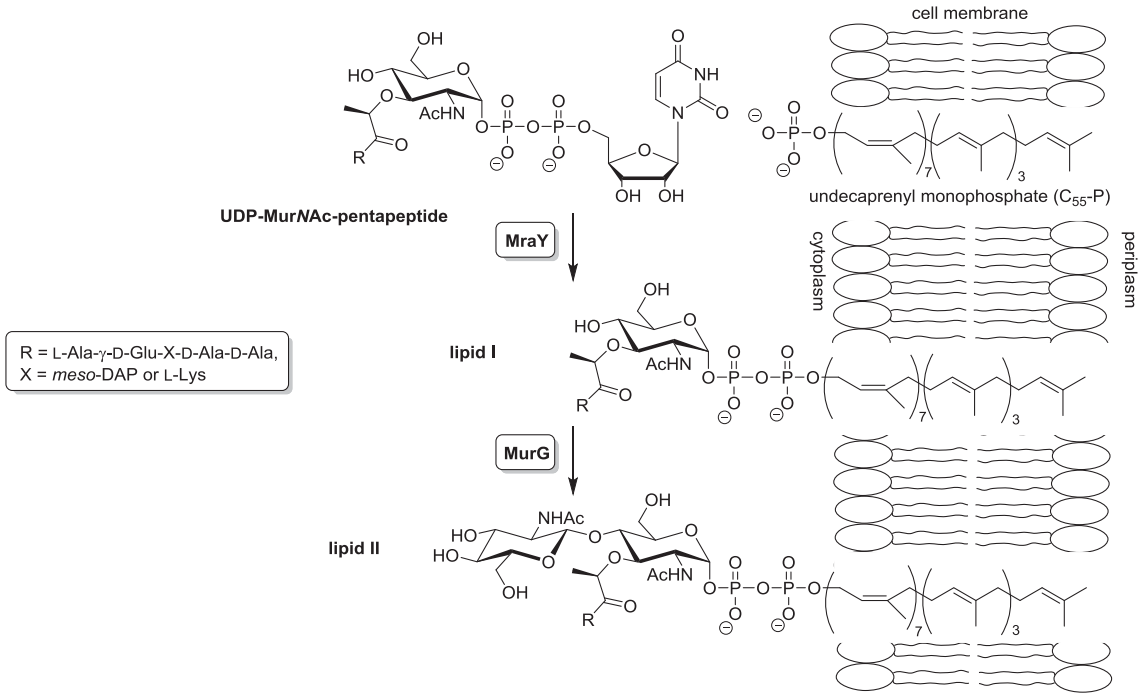

Figure 2.3: Biosynthesis of the peptidoglycan precursors lipid I and lipid II (adapted from: A. Matsuda et al., *J. Med. Chem.* **2011**, *54*, 8421).[52]

Early mechanistic studies showed that MraY utilizes the two substrates undecaprenyl phosphate and UDP-Mur*N*Ac pentapeptide. The transferase activity is fully reversible, and it also catalyzes an exchange between UMP and UDP-Mur*N*Ac pentapeptide, suggesting a two-step mechanism: (1) the formation of an enzyme-substrate complex under the release of UMP and (2) further reaction to lipid I.[53-55] In vivo, this reversible two-step reaction is coupled to the subsequent lipid II formation, which is catalyzed by the transferase MurG.[56] Although the encoding gene *mraY* had already been identified in 1991[57] and a first topology model suggesting MraY as an integral trans-membrane protein had been postulated in 1999,[58] it was not until 2004 that MraY was placed in the focus of scientists as an attractive target for antibiotic research. Due to the significant overexpression, purification, and characterization of MraY by *Mengin-Lecreulx* and coworkers,[56] a first model for the active site could be developed.[59] In 2011, *Bernhard* and coworkers were able to express MraY by cell-free methods.[60] Recently, the crystal structure of MraY was reported by Chung et al.[61] Although the binding and inhibition mechanism of MraY has still not been completely understood, this achievement will pave the way for the discovery of potent inhibitors. As MraY is essential for bacterial viability and only present in bacteria, compounds inhibiting this enzyme are quite attractive for the development of

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antibiotics. As the inhibition of lipid I formation represents a whole new target, there are no MraY inhibitors on the clinical market yet.

There are different natural products known which have the ability to inhibit MraY. A quite interesting group is represented by nucleoside antibiotics.[51,62] This class of structurally complex compounds shares a uridine-based motif. The nucleoside building block is connected to structurally different scaffolds via the 5'-C, depending on the compound set (Figure 2.4).

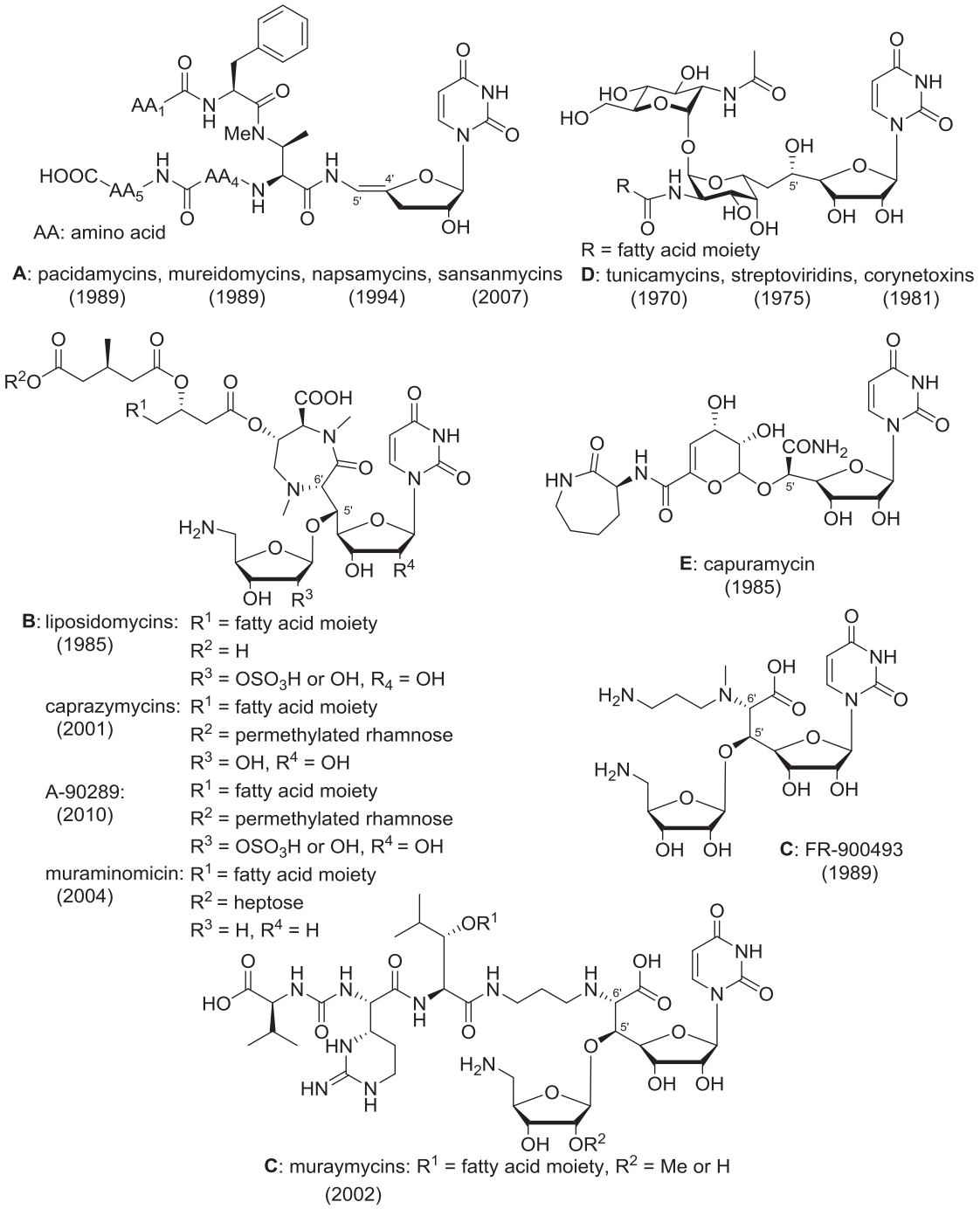

Figure 2.4: Structures of the nucleoside antibiotic groups **A**-**E** inhibiting MraY. The year of the first isolation is given in parentheses.

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